C5 Cont

and/or heparin with amyloid precursor protein (APP) of said patient, with the proviso that said agent is not EDTA.

C6

31. (amended) A method according to claim 30, wherein said zinc-binding agent is selected from sodium citrate, 1,2-diethyl-3-hydroxypyridin-4-one, and 1-hydroxyethyl-3-hydroxy-2-methylpyridin-4-one.

REMARKS

I. Status of the Claims

Upon entry of this amendment, claims 28-33 are pending. No new matter has been added.

II. Requirement for Restriction

The Examiner has maintained and made final the requirement for restriction between Group I, drawn to a method for treating Alzheimer's disease, now represented by claims 28-33, and Group II, drawn to a method of screening compounds, represented by claim 27. Applicants maintain that no serious burden would exist in examining both Groups. Nevertheless, applicants have rendered moot the requirement for restriction by canceling claim 27. Applicants of course reserve their right to file the non-elected subject matter in one or more continuing or divisional applications.

III. Specification and Drawings

Applicants will provide formal drawings when required by the Examiner. Applicants have amended the specification at pages 2, 7, and 32 to correct the obvious

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typographical errors pointed out by the Examiner. A section entitled Brief Description of the Drawings has been added at page 12, per the Examiner's suggestion. Applicants choose not to adopt the Examiner's suggestion to modify the list of references at pages 36-38.

Clarification was requested of the sentence in the specification that appears at page 10, lines 7-9. This text is clear to a person of ordinary skill in this art as the concept of compartments is basic in biology. Compartments are enclosed spaces in which movement between the inside and outside is restricted. The compartments described in the present specification refer to the contents of the cell that are enclosed by the cytoplasmic membrane (the intracellular compartment) and the material excluded from this compartment but within the confines of the body (the extracellular compartment). Additional description of these compartments is found in the present specification, for example, at page 9, line 28 to page 10, line 3 and page 10, lines 23-28. Accordingly, the Office is requested to withdraw this objection to the specification.

IV. Rejections Under 35 U.S.C. § 112, First Paragraph

Claims 28-33 were rejected under 35 U.S.C. § 112, first paragraph for allegedly failing to provide an enabling disclosure. The Examiner has stated that Applicants' described basis for their claimed method is "theoretical." Office action at page 6. In addition, the Examiner finds the description "controversial, confusing and not supported by experimental data, which makes certain statements lacking scientific credibility." *Id.* The Examiner has referred to page 6, lines 21-30 as one such "controversial" passage,

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owing to the disclosure of zinc loading experiments and the effect of zinc loading on abnormal cleavage of APP. *Id.* Applicants respectfully traverse this rejection.

Applicants have submitted a copy of a declaration by Professor Colin L. Masters filed under 37 C.F.R. § 1.132 in parent application number 08/757,537. It is clear from paragraph 7 of the Masters declaration that zinc loading experiments in rats resulted in elevated levels of full length APP and reduced levels of soluble APP compared to control. A similar experiment with aluminum produced no such changed APP levels compared to control. Therefore, since applicants have supported their disclosure of the effect of zinc on APPase-mediated cleavage with actual experimental evidence, withdrawal of this aspect of the rejection is requested.

The Examiner has also questioned enablement for the role of heparin in APP-ase mediated cleavage because "heparin function . . . is . . . not supported by any references to any scientific publication (page 7, lines 21-27)." *Id.* Applicants note that their disclosure is presumptively accurate and enabled. There is no need for applicant to document or prove the accuracy of their disclosure unless the Examiner supports her reason to doubt the accuracy with acceptable evidence or reasoning. MPEP, 8th Edition, § 2164.04. Applicants submit that a mere reference to the absence of scientific publications is insufficient reason for doubting the accuracy of their disclosure.

The Examiner has further stated that undue experimentation would be required to practice the full scope of the invention as claimed. Office action at page 7. She has noted, *inter alia*, the absence of working examples. *Id.* Working examples are not required, but are simply one way to provide information sufficient to comply with § 112, first paragraph. It is respectfully submitted that the present specification provides

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adequate teaching to support the claimed invention. The identical issues raised in the present Office Action were raised and overcome in the parent application.

Applicants further draw the Examiner's attention to the declaration of Professor Masters filed in the parent application. Paragraph 6 of said declaration shows clearly that in an acceptable animal (TG2576 mouse) model, amounts of sedimentable Aβ peptides were reduced (relative to untreated controls) following treatment with clioquinol, a known metal ion chelator. In addition the treated mice showed improved behavior. Their startled responses returned and their abnormal "spinning" movements, typical of diseased transgenic mice, ceased. Since Applicants have provided data in support of their presumptively enabled disclosure, withdrawal of this aspect of the rejection is also requested.

The Examiner has also questioned enablement for treating Alzheimer's disease. The Masters declaration at paragraph 6 makes it clear that one can easily dose symptomatic mice, a recognized animal model for Alzheimer's disease, and readily observe successful treatment by cessation of typical symptoms. There is no need to include in a specification that which is well-known and already available to the public. MPEP § 2164.05(a). Dosing and relief or disappearance of symptoms are part of any treatment for any ailment. Withdrawal of this aspect of the rejection is requested.

Finally, the Office has stated that the specification is confusing, and has made specific reference to the passage at page 7, line 29 to the effect that "low zinc concentrations (above about 1 μM)" is unclear. Note that this sentence has been amended to correct the obvious error of referring to "μm" or micrometers, to "μM" or micromoles. Further, this sentence describes that not only are the protective effects of

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heparin abolished at low zinc concentrations of about 1 μM, but are also abolished at zinc concentrations above 1 μM. Accordingly, the Office is requested to withdraw this ground of rejection.

V. Rejections Under 35 U.S.C. § 112, Second Paragraph

Claim 28 was rejected as indefinite under 35 U.S.C. § 112, second paragraph. The Examiner views the phrase "a therapeutically effective amount of an agent" as unclear because "[n]o such 'effective amount' is indicated" in the claim or specification. Office action at page 8. Applicants respectfully traverse this rejection and direct the Examiner's attention to MPEP § 2173.05(c), subsection III. The proper test is whether or not one skilled in the art could determine specific values for the amount based on the disclosure. When the phrase is read in light of the preamble to claim 28 and the supporting disclosure, it is apparent that the amount is effective to treat Alzheimer's disease. As can be seen from the Masters declaration, paragraph 6, one can readily observe from the behavior of the host when the amount is effective. Determination of suitable dosages is a matter of routine experimentation once a drug is proven effective. Withdrawal of this rejection is respectfully requested.

The Examiner also requests clarification of the phrase "interaction within the central nervous system." It is axiomatic that claim definiteness is not determined in a vacuum. One must analyze the claim in light of the specification, the teachings of the prior art, and the claim interpretation given the claim by one of ordinary skill in the pertinent art at the time the invention was made. MPEP § 2173.02. Applicants' specification clearly sets forth at page 7 the manner in which the claimed interaction is

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to be interpreted. The divalent or trivalent cations and/or heparin bind heparin binding sites on APP. This binding is an interaction, i.e., an acting on each other.

Claim 31 is rejected for being an improper Markush claim because of the language "selected from . . . and." Apparently, the Office's position is that Applicants have used Markush language and the wrong conjunction "and." An inventor using a Markush group may choose to recite the conjunctive (and) or the disjunctive (or):

> When materials recited in a claim are so related as to constitute a proper Markush group, *they may be recited in the conventional manner, or alternatively.* For example, if 'wherein R is a material selected from the group consisting of A, B, C **and** D is a proper limitation, then wherein R is A, B, C **or** D shall also be considered proper.

M.P.E.P. § 2173.05(h) (emphasis added). Both are considered proper.

However, in the present claims Applicants have not used "Markush" language. Markush language is only one of many accepted forms of claim language: "One acceptable form of alternative expression, which is commonly referred to as a Markush group, recites members as being "*selected from the group consisting of A, B and C.*" See Ex parte Markush, 1925 C.D. 126 (Comm'r Pat. 1925)." *M.P.E.P.* § 2173.05(h) (emphasis added). A Markush group follows the format of the italicized phrase. Here, however, the italicized phrase is absent from the present claims. Thus, the present claims are not "Markush" groups and are not within the ambit of the Examiner's reasoning.

The Office must allow alternative expressions if one of ordinary skill in the art would have been reasonably apprised of the scope of the claims: "Alternative expressions are permitted if they present no uncertainty or ambiguity with respect to the question of scope or clarity of the claims." *M.P.E.P.* § 2173.05(h). The Office has

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pointed to no uncertainty or ambiguity regarding the scope or clarity of the present claims. Thus, Applicants respectfully submit that the Office failed to meet its burden of establishing a prima facie case for indefiniteness.

The phrase (**A**) "X is selected from A, B, and C" is proper language and clearly describes a claimed invention. For example, X may be A; A and B; or two As, two Bs and a C, as well as all other permutations. Moreover, X may even be A and Y, where Y is not embraced by A, B, and C. Phrase (**A**) is open-ended and clear. By analogy, Applicants' claim language is clear, and the Office has shown no legal basis for requiring Applicants to change it. Applicants respectfully request that this ground for rejection be withdrawn.

Claims 29, 30, 32, and 33 were rejected for depending upon claims rejected for indefiniteness. Since independent claims 28 and 31 are not indefinite for the reasons set forth above, Applicants respectfully request that this ground for rejection be withdrawn as well.

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VI. Rejection Under 35 U.S.C. § 102(b)

Claims 28-31 and 33 were rejected under 35 U.S.C. § 102(b) as anticipated by Cardelli et al. (J. Am. Geriatr. Soc. 1985, 33, 548-60). The Examiner views the disclosure of EDTA in the reference as anticipatory. Applicants have amended claims 28 and 31 to exclude EDTA. The rejection is now moot. Withdrawal of this rejection is requested.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P.

Dated: December 10, 2001

By: Charles E. Van Horn

Charles E. Van Horn
Reg. No. 40,266

Enclosure: Rule 132 declaration of Professor Colin L. Masters.

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VERSION WITH MARKINGS TO SHOW CHANGES MADE PURSUANT TO 37 C.F.R. 1.121(c)(1)(ii)

IN THE SPECIFICATION

At page 2, first complete paragraph:

There is a need for an assay which is of predictive and diagnostic value in monitoring Alzheimer's disease and for any therapeutic interventions therein. In accordance with the present invention, it has now been discovered [.at] that processing of circulatory APP is altered in Alzheimer's disease thus providing a basis for an assay for the disease. Furthermore, from work leading up to the present assay, an improved means of treating Alzheimer's disease has been discovered based on modulating the interaction between divalent cations and/or heparin and APP.

At pages 7 and 8:

By modulating the levels of divalent cations or heparin or any other moiety which can bind the heparin binding sites on APP (residues 318-331 and around residues 98-105) or any other binding site on APP capable of binding these moieties (such as additional zinc or heparin binding sites on APP), the range, type and/or extent of APP cleavage can be altered such that incorrect protease-mediated processing of APP can be reduced or inhibited. By "modulate" is meant the alteration of the availability of divalent cations and trivalent cations or heparin or any other moiety which can bind the heparin binding sites on APP (residues 318-331 and around residues 98-105) or any other binding site on APP [ATP] capable of binding these moieties (such as additional zinc or heparin binding sites on APP) to bind to APP prior to or simultaneously with APPase-mediated cleavage. It has been found that zinc (Zn^{2+}) binds to APP at a specific and

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saturable binding site. The zinc binding site on APP was identified by enzymatic digestion of purified APP695-fusion protein coupled to Zn^{2+} chelating sepharose. The synthetic peptide representing about residues 181-200 of APP, situated between the cysteine rich and negatively charged domains of the protein, was shown to bind zinc in a specific and saturable manner. The intimate involvement between APP and zinc is strongly suggestive of a role of zinc in APP processing: APP binds heparin (in a manner analogous to FGF). Heparin has been shown to protect APP from proteolytic digestion [digesion], as exemplified using the proteolytic enzyme trypsin. Heparin concentration as low as 100 nM cause a marked reduction in the rate and degree of brain APP degradation by trypsin. The brain contains a number of heparin or heparin sulphate containing proteins and thus the interaction of heparin with APP may stabilise APP from proteolytic degradation in-vivo. It has also been found that zinc affects [effects] the kinetics of heparin binding to APP, and may increase APP affinity for heparin 5 to 10 fold. Surprisingly, at low zinc concentrations (above about 1 μM [μm]) the protective effects of heparin are abolished. This finding indicates that aberrant zinc levels in-vivo, in the brain intracellular and/or extracellular milieu [milieu], may promote aberrant APP proteolytic processing giving rise to the amyloid protein, and subsequently Alzheimer's disease and other disorders associated with amyloid deposition in the brain.

At page 12:

BRIEF DESCRIPTION OF THE DRAWINGS

[In the Figures:]

FIGURE 1 is a photographic representation showing immunoblots comparing Alzheimer's disease and age matched control plasma APP. Plasma heparin-Sepharose

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eluates (65 µg) were analysed by 8.5% (w/v) SDS polyacrylamide gel electrophoresis and immunoblotting with MAb 22C11 which recognises an amino-terminal epitope (see Example 1). The relative molecular mass of standard protein markers (Rainbow Standards, Amersham, UK) are shown on the left. APP immunoreactive bands of 130, 110 (a doublet), 65 and 42 kDa are indicated by arrows to the right. Only the relative abundances of the 130 and 42 kDa APP forms, as in the sample illustrated, could visibly discriminate between Alzheimer's disease compared to (Figure 1A) non-demented elderly controls and (Figure 1B) normal young control populations.

At pages 32-33:

EXAMPLE 6

ADMINISTRATION OF ZINC IN ALZHEIMER'S DISEASE (AD)

The subjects from Example 3 were studied.

The healthy volunteers suffered no ill effects from the zinc supplementation.

The two AD volunteers became [because] acutely unwell while on zinc supplementation. They both suffered a severe loss of cognitive function with mini-mental state examination (Folstein et al., 1975) scores deteriorating from moderately demented levels to unrecordable. Eye movement abnormalities and general levels of self care worsened over the period of supplementation. This response was consistent with a neurotoxic response to the zinc supplementation. When zinc supplementation was ceased, cognitive function returned to the previous levels within two weeks.

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IN THE CLAIMS

28. (amended) A method for treating Alzheimer's disease [Disease] in a patient comprising the step of subjecting said patient to a therapeutically effective amount of an agent which is capable of crossing the blood brain barrier, wherein said agent modulates the interaction within the central nervous system between a divalent or trivalent cation and/or heparin with amyloid precursor protein (APP) of said patient, with the proviso that said agent is not EDTA.

31. (amended) A method according to claim 30, wherein said zinc-binding agent is selected from sodium citrate, [EDTA,] 1,2-diethyl-3-hydroxypyridin-4-one, and 1-hydroxyethyl-3-hydroxy-2-methylpyridin-4-one.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Masters, C L *et al*	Docket:	9287Z
Serial No. :	08/757,537	Group Art Unit:	1645
Filed :	26 November, 1996	Examiner:	Duffy, P
For :	A METHOD FOR ASSAYING AND TREATING ALZHEIMER'S DISEASE		

Honorable Commissioner of
Patent and Trademarks
Washington, D.C. 20231

DECLARATION PURSUANT TO 37 C.F.R. §1.132

I, Professor Colin L Masters, hereby declare as follows:

1. I am currently the Professor and Chairperson of the Department of Pathology at The University of Melbourne, Parkville, Victoria, Australia. My Curriculum Vitae is attached hereto as Exhibit I.

3. I have published extensively in the area of neurodegenerative disease. A list of my publications is included in my Curriculum Vitae (Exhibit I).

4. I am an inventor of subject matter contained and described in United States Patent Application Serial No. 08/757,537 filed on 26 November, 1996 (hereinafter referred to the "APPLICATION"). The APPLICATION is directed *inter alia* to a method for treating Alzheimer's disease by modulating divalent cation and/or heparin interaction with amyloid precursor protein (hereinafter referred to as "APP"). In particular, claim 15 of the APPLICATION provides:

> "[a] method for modulating the level and/or processing of APP in a patient with Alzheimer's disease, comprising subjecting said patient to a means that modulates

divalent or trivalent cation and/or heparin interaction with APP".

5.. Alzheimer's disease is a progressive dementia characterised by the deposition of amyloid plaques in the intracellular and extracellular compartments of the cerebral cortex. The main constituent of the amyloid plaque is a peptide referred to as Aβ which results from incorrect processing of APP. The amyloidocentric pathway leading to Alzheimer's disease is shown in Exhibit II.

The amyloid plaque comprises extracellular or perivascular congophillic deposits of aggregated Aβ with a high content of β pleated sheet secondary structure. The amyloid plaque is the end result of a process of Aβ oligomerisation, fibral formation, aggregation and precipitation occurring in several states wherein each state potentially has a different impact on surrounding neurones. It is proposed that soluble forms of Aβ oligomers represent the toxic species. The oligomers then aggregate into protofibrillar structures which are first seen as precipitates in defuse amyloid plaques; this progresses with dystrophic muriate formation both within the neuropil and around dense crystalloid precipitate of amyloid cores.

In Alzheimer's disease, processing of APP creates a high ratio of a "long" form of Aβ comprising 42 amino acids referred to as Aβ42 relative to a "short" form of Aβ comprising 40 amino acids referred to as Aβ40. The more insoluble long Aβ42 form is a primary constituent of the amyloid plaque.

APP is a transmembrane protein which is found in most cell types including neuronal and glyal cells. At critical points in its biogenesis, APP is subjected to enzymatic protolytic cleavage which in concert generate the Aβ peptides. This shown in the diagram in Exhibit III. These enzymes, termed secetretases, release the APP from the cell membrane and thereby effect the proportion of the protein that remains on the cell surface or is released into the extracellular milieu. The Aβ peptides encompass part of the hydrophobic transmembrane domain. The cleavage sites of the γ-secetretases are important since the length of the hydrophobic tail of the Aβ peptide is a critical factor determining its aggregation and toxicity. Thus, Aβ40 is the species most often identified in the non-neuronal cells and has less tendency to aggregate then Aβ42.

Once released from the cell, Aβ peptides aggregate into amyloid fibrils. The rates of deposition and clearance of Aβ from the brain is critical to determinants in establishing disease.

Accordingly, it is clear that modulating the processing of APP effects the extent of Aβ formation and, hence, amyloid plaque formation. As a result, modulating processing of APP can be effective in treating Alzheimer's disease.

6. In conjunction with my scientific collaborators, I conducted studies in a recognised animal model for Alzheimer's disease. This animal model is the TG2576 mouse available from Merk. In this study, 12 month old transgenic animals with established Alzheimer's disease pathology were treated with either a placebo or a compound known as clioquinol. This compound is a known metal ion chelator. The placebo and the clioquinol were administered orally every day for three months. The mice were then sacrificed and the insoluble Aβ amyloid plaques in the brain measured by Western blot. The results are shown in Exhibit IV. Using this Western blot analysis, sedimentable Aβ was determined in μg/g. The diamond shapes on the left hand side of the graph (Exhibit IV), show mice given a placebo. These are the untreated controls. The hexagonal shapes on the right hand side of the graph show the results of mice given 20 mg/kg of clioquinol. The mean values are statistically significant were the amount of sedimentable Aβ in the clioquinol treated mice is less than the Aβ in the untreated controls. Animals treated with clioquinol were, in addition, observed to be behaviourally improved. There startled responses returned and abnormal "spinning" movements, typical of diseased transgenic mice, ceased.

It is concluded, therefore, that using a metal chelator which modulates divalent and trivalent metal cation interaction with APP resulted in a decrease in the amount of Aβ and resulted in an improved health effect on mice.

7. In another experiment, we investigated the effects of oral zinc ingestion upon brain APP in rats. The graph in Exhibit V shows rats supplemented with a zinc (elemental zinc 140 ± 50 mg/kg body weight over 7 days; approximately 3 fold in excess of the human daily recommended daily allowance). The APP:GAPDH mRNA ratio was significantly increased

compared to rats supplemented with aluminium or not supplemented at all. The protein levels of APP were assayed in the same animals. In the untreated animals, APP in the soluble fraction of brain homogenates (APPs) was approximately 5-fold more abundant than the full length APP (APP_{FL}) found in the palatable fraction of the brain homogenates, indicating that the processing of APP exists at an equilibrium that favours the cleavage of full length APP. Following exposure to zinc, there was a significant reduction of APPs and a significant elevation in APP compared to control. Compared to untreated control animals there is no change in APPs or APP_{FL} seen in the brains of rats that ingested aluminium for the same period.

These data indicate that exposure to nutritional zinc can alter the transcription and processing equilibrium of APP favouring stabilisation of APP_{FL}.

8. It is my considered scientific opinion that these data support the claim that modulating the level and/or processing of APP by a means which modulates divalent or trivalent cation and/or heparin interaction with APP assists in the treatment of Alzheimer's disease.

I declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardise the validity of the APPLICATION or any patent issuing thereon.

Date: July 8, 99

Professor Colin L Masters

COLIN L MASTERS

Curriculum Vitae

DATE AND PLACE OF BIRTH: February 5, 1947, Perth, Australia

CITIZENSHIP: Australian

ADDRESS:

Work: Department of Pathology
The University of Melbourne
Parkville, Victoria 3052
Tel: +61 3 9344 5868
FAX: +61 3 9344 4004
e-mail: c.masters@pathology.unimelb.edu.au

Home: 171 Gold Street
Clifton Hill, Victoria 3068
Tel: +61 3 9489 2951

FAMILY STATUS: Married, three children.

CURRENT APPOINTMENTS:

Professor and Head, Department of Pathology, The University of Melbourne (1989).

Chief of Neuropathology Laboratory (1989) and Director of Research Laboratories (1997), Mental Health Research Institute of Victoria.

Consultant in Pathology, The Royal Melbourne Hospital (1989) and Teaching and Research, The North-Western Health Care Network, Melbourne (1997).

Consultant in Neuropathology, Victorian Institute of Forensic Medicine (1989).

Consultant, The Walter and Eliza Hall Institute of Medical Research (1992).

TERTIARY EDUCATION:

1964	Commenced undergraduate studies at the University of Western Australia.
1967	B.Med.Sci.(Physiology) (First Class Honours) University of Western Australia.
1970	M.B., B.S., University of Western Australia.
1977	M.D., University of Western Australia.

ACADEMIES OF SCIENCE AND COLLEGES OF PATHOLOGISTS:

Fellow, Australian Academy of Science (1999)

Fellow, Royal College of Pathologists (1986)

Fellow, The Royal College of Pathologists of Australasia (1989)

PROFESSIONAL AND ACADEMIC HISTORY:

1968	Research Student examining the pathology of unconventional virus diseases of the nervous system. Departments of Pathology and Microbiology, University of Western Australia.
1971	Resident Medical Officer, Royal Perth Hospital, Perth.
1972-1974	Research Fellow, Department of Pathology, University of Western Australia, Dr. Michael Alpers, Dr. Byron Kakulas. (Supported by Faculty of Medicine Research Fellowship).
1975	Medical Registrar, Sir Charles Gairdner Hospital, Perth (General Medicine, Immunology and Neurology).
1976	Research Fellow, Department of Pathology, University of Western Australia, Perth. (Supported by Faculty of Medicine Research Fellowship).
1976-1977	Research Fellow, Neuropathology, Massachusetts General Hospital, and Harvard Medical School, Boston, Dr. E.P. Richardson, Jr.
1977-1980	Visiting Scientist, Laboratory of Central Nervous System Studies, National Institutes of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda. Dr. D. Carleton Gajdusek.
1980-1981	Guest Professor and Humboldt Fellow, Institute of Neurobiology, University of Heidelberg, Federal Republic of Germany, Dr. Melitta Schachner.
1981-1988	Principal Research Fellow, National Health and Medical Research Council of Australia, Department of Pathology, University of Western Australia.
1981-1988	Clinical Assistant (Research), Department of Neuropathology, Royal Perth Hospital.
1988	Overseas study leave. Center for Molecular Biology, University of Heidelberg.

MEMBERSHIPS OF SOCIETIES:

Alzheimer's Society of Victoria
American Association of Neuropathologists
American Society for Cell Biology
American Society for Microbiology
Australian and New Zealand Society for Neuropathology
Australian Association of Neurologists
Australian Neuroscience Society
Australian Society for Medical Research
International Academy of Pathology, Australian Division
International Society of Neuropathology
Pathological Society of Great Britain and Ireland
Society for Neuroscience

EDITORIAL BOARDS:

Alzheimer's Disease and Associated Disorders - An International Journal (1993 -)
Alzheimer's Reports (1998 -)
Alzheimer's Research (1996 - 1998)
American Journal of Alzheimer's Disease (1997 -)
Amyloid - A Journal of Experimental and Clinical Investigation (1993 -)
Archives of Neurology, International Advisory Committee (1996 -)
Australian Journal on Ageing (1994 -)
Brain Pathology (1990-1995)
Brain Research (1994-)
Dementia and Geriatric Cognitive Disorders (1989 -)
European Journal of Neurology (1994 -)
Experimental Neurology (Neurodegeneration section) (1994 -)
Journal of Clinical Neuroscience (1994 -)
Journal of Tropical and Geographical Neurology (1990 - 1993)
InScight Editorial Board (1998 -)
Molecular Psychiatry (1995 -)
Neurobiology of Aging (1990 - 1993)
Neuropathology (1997 -)
Neuroscience News (1997 -)
Research and Perspectives in Alzheimer's Disease (Fondation IPSEN) (1990 -)

ADVISORY AND EXECUTIVE COMMITTEE MEMBERSHIPS:

Alzheimer's Disease International. Medical and Scientific Advisory Panel.

Alzheimer's Disease and Related Disorders Association of Australia. Scientific Advisory Committee.

Alzheimer's Research Trust (Cambridge, UK). Scientific Advisory Board.

Animal Experimentation and Ethics Committee, the University of Melbourne.

Anti-Cancer Council of Victoria. Medical and Scientific Committee.

Association de Lutte contre les Maladies à Prions. International Scientific Committee.

Bone Marrow Research Laboratories Advisory Committee. The Royal Melbourne Hospital Research Foundation.

Foundation for the Detection of Genetic Disorders. Board of Management.

International Conference on Alzheimer's Disease and Related Disorders. International Scientific Advisory Committee.

International Symposium on Amyloidoses. Nomenclature Committee.

Melbourne Neuromuscular Research Centre, St Vincent's Hospital. Committee of Management.

Mental Health Research Institute of Victoria. Scientific Advisory Committee.

National Health and Medical Research Council. Assessor for Project Grants and Training Awards; Regional Grants Interview Committee.

National Health and Medical Research Council. Network of Brain Research into Mental Disorders. Executive Committee.

National Pituitary Hormones Advisory Council. Research Committee.

National Serology Reference Laboratory. Scientific Advisory Committee (Chairman) and Management Committee.

PRANA Corporation. Chairman, Scientific Advisory Board.

Research Group on Dementia, World Federation of Neurology. Executive Committee.

Royal College of Pathologists of Australasia. Victorian State Committee.

Van Cleef/Roet Centre for Nervous Diseases, Monash University. Advisory Board.

HONORS, PRIZES, INVITED LECTURES, AND OTHER SPECIAL SCIENTIFIC RECOGNITION:

1966 National Heart Foundation, Vacation Scholar Scholarship, Department of Physiology, University of Western Australia.

1968 Marion Margaret Bergin Memorial Prize in Pathology.
Boots Proprietary Limited Prize in Medical Microbiology.

1970 Queen Elizabeth II Coronation Gift Fund Trust Prize (in Paediatrics). John Lindsay Taylor Memorial Prize (in Gynaecology).

1980 Award from Stiftung zur Bekämpfung neuroviraler Krankheiten (Hamburg).

1983 Faculty Member for course on dementias, American Academy of Neurology (San Diego).

Member of the Work Group of Department of Health and Human Services Task Force on Alzheimer's Disease: Etiology and Pathogenesis (Washington, D.C.).

1984 European Molecular Biology Organization Workshop on Slow Virus Diseases (Edinburgh).

1985 *Invited lectures*: Spring Meeting of the Institute of Genetics, University of Cologne (Cologne); NH&MRC Workshop on Aging and Age Related Disability (Sydney); World Congress of Neurology, Symposium on Neurovirology (Hamburg).

1986 *Invited lectures:* CIBA Symposium on Selective Neuronal Death (London); International Symposium on Amyloidosis (Groningen); Princess Liliane Foundation Symposium on the Aging Brain (Brussels); Second International Congress on Unconventional Virus Infections of the Nervous System (Paris).

1987 Awardee of Senior Technical Advisory Recruitment (STAR) Programme, Human Resources Development, United Nations Development Programme (Beijing, People's Republic of China).

Presidential Award from the International Association of Gerontology.

Invited lectures: Workshop on Research in Aging, Commonwealth Department of Veterans' Affairs (Sydney); Colloquium on Alzheimer's Disease, International Society for Neurochemistry and American Society for Neurochemistry (Venezuela); National Institute of Mental Health Workshop on the Epidemiology of Alzheimer's Disease (Bethesda, Maryland); CIBA Symposium on Unconventional Viruses and the Nervous System (London);

Dahlem Workshop on the Aetiology of Dementia of the Alzheimer Type (Berlin).

1988 Dr. Gunther Buch prize (with Konrad Beyreuther).

Robert Pfleger Prize (with Konrad Beyreuther).

Invited lectures: Fondation pour l'Etude du Systéme Nerveux Central et Peripherique study group on "Molecular Genetic Mechanisms in Neurological Disorders" (Geneva); International Symposium on Alzheimer's disease (Kuopio, Finland); Paulo Foundation International Symposium on Pathobiology of Alzheimer's Disease (Helsinki); Conference on Dynamics of Protein Development and Function (Heidelberg); ZMBH-Forum on 'Neurobiology of Development and Disease' (Heidelberg); "Frontiers of Research in Neuroscience", Medical Bioscience Symposium (Kumamoto); Fondation IPSEN Colloques medicine et recherche: Genetique et Maladie d'Alzheimer (Paris); Cold Spring Harbor Banbury Center: Molecular Biology of Alzheimer's Disease (Cold Spring Harbor, New York).

1989 *Invited lectures*: International Study Group on the Pharmacology of Memory Disorders Associated with Aging (Zurich).

1990 American Academy of Neurology, Potamkin Prize (with Konrad Beyreuther).

1991 Max Planck Research Award from the Alexander von Humboldt Foundation (with Konrad Beyreuther).

Invited lectures: International Society for Neurochemistry (Sydney)

1992 *Invited lectures*: Third International Conference on Alzheimer's Disease and Related Disorders (Padua, Italy); Australian Association of Neurologists (Melbourne); Society for Neuroscience (New Orleans); Alzheimer's Association Australia (Adelaide); International Conference on Aluminium and Health (Tampa, Florida); Sandoz Lectures in Gerontology (Basle, Switzerland)

1993 Chairman, Fondation IPSEN Meeting on "βA4 Amyloid Protein Precursor in Development, Aging and Alzheimer's Disease" (Lyon).

Fellow, Brain-Behaviour Research Institute, LaTrobe University.

Invited lectures: FASTS Australian Neuroscience Lecturer (Melbourne); Alzheimer's disease: Progress for the next decade. Ramon Areces Foundation (Madrid); World Congress of Gerontology (Budapest); International Symposium on Amyloidosis (Kingston, Ontario); International Symposium on Alzheimer's Disease (Tokyo); World Congress of Neurology (Vancouver); International Congress of Clinical Chemistry (Melbourne)

1994 *Invited lectures*: International Conference on Aluminium and Health (Tampa, Florida); International Conference on Alzheimer's Disease and Related Disorders (Minneapolis); National Conference, Alzheimer's Association Australia (Sydney); Deidesheimer Gespräch (Heidelberg); Biotech 2000 - Symposium (Seoul); Collegium Internationale Neuro-Psychopharmacologicum (Washington); European Society of Neurochemistry (Jerusalem).

1995 Convenor, International Workshop on Creutzfeldt-Jakob Disease (Melbourne).

KJ Zülch Prize (with Konrad Beyreuther) from the Gertrud Reemtsma Stiftung of the Max Planck Society (Cologne).

Invited lectures: World Federation of Neurology (Dementia) and Society of Neuroscience in Africa (Marakesch); CIBA Foundation Meeting on Amyloid (Portugal); International Psychogeriatric Association (Sydney).

1996 WHO Consultation on Clinical and Neuropathological Characteristics of the New Variant of Creutzfeldt-Jakob disease and other human and animal transmissible spongiform Encephalopathies (Geneva).

Invited lectures: International Conference on Prion Diseases (Paris); Cold Spring Harbor Symposium on Quantitative Biology: Function and Diseases of the Nervous System (New York); Convenor of Symposium for the Collegium Internationale Neuro-Psychopharmacologicum (CINP) on the Molecular Basis of New Therapeutic Strategies for Alzheimer's Disease (Melbourne); International Conference on Alzheimer's Disease and Related Disorders: Round table on Alzheimer's Disease: Convergent Mechanisms and Divergent Therapies (Osaka); Heidelberger Akademie der Wissenschaften. Epithelial Cells and Neuronal Organization/Plasticity and Neuronal Degeneration (Heidelberg); Invited Lecture, Australian Association of Gerontology (Melbourne); Australian Society for Microbiology (Melbourne); Royal College of Pathologists of Australasia (Sydney); Australian Academy of Science (Canberra).

1997 King Faisal International Prize in Medicine (with Konrad Beyreuther and James Gusella)

George S Christie Lecture, Australasian Society for Experimental Pathology.

Erna Struckmann Lecture, Centre for Molecular Biology, The University of Heidelberg.

Chairperson, WHO Consultation on Medical and other Products in Relation to Human and Animal Transmissible Spongiform Encephalopathies (Geneva).

Invited lectures: Australian Association of Neurologists (Sydney); International Society for Neurochemistry, Advanced School of Neurochemistry (Amherst College, Massachusetts); World Congress of Gerontology (Adelaide/Hawaii).

1998 Alois Alzheimer Award.

Invited lectures: Australian Association of Neurologists (Brisbane); Haematology Society of Australia (Sydney); Australian Red Cross Blood Service (Melbourne); Combined Biological Sciences Meeting (Perth); Human Genetics Society of Australia (Melbourne); International Conference on Alzheimer's disease (Amsterdam); 150th Anniversary of the Royal Melbourne Hospital.

1999 WHO Consultation on Diagnostic Procedures for Transmissible Spongiform Encephalopathies and WHO Consultation on Caring for Patients and Hospital Infection Control in Relation to Human Transmissible Spongiform Encephalopathies (Geneva).

Invited lectures: Keystone Symposia, Molecular Mechanisms in Alzheimer's Disease (Taos); WHO/IPSEN meeting on Genetic Resistance to Disease (Venice); Australian Society for Geriatric Medicine (Perth); Royal College of Pathologists of Australasia (Melbourne); Australasian Association of Clinical Biochemists (Melbourne)

Research Profile and Achievments

Colin Masters began his research career as a 1966 summer vacation student working with Evan Morgan (Physiology, UWA) on the placental transfer of plasma proteins. His interests in neuroscience stem from this time when he then took the opportunity to pursue a Bachelor of Medical Science degree with Brian Johnstone and Judith Laszlo (Physiology, UWA) resulting in the first demonstration of brain-stem evoked responses to auditory stimuli in humans. Toward the end of 1967, MacFarlane Burnet gave a lecture in Perth on kuru. This, together with the connections that Byron Kakulas (Pathology, UWA) and Michael Alpers (Microbiology, UWA) had established with D. Carleton Gajdusek and Clarence J. Gibbs at the NIH, led to the still ongoing study of the transmissible spongiform encephalopathies (prion diseases). These studies began with the pathologic evaluation of preclinical disease, and continued with the nature of spongiform change (with EP Richardson, Massachusetts General Hospital, Harvard Medical School), the epidemiology of Creutzfeldt-Jakob disease, the familial occurrence of these diseases, and the identification of a special subgroup (the Gerstmann-Sträussler-Scheinker [GSS] syndrome) in which abundant amyloid deposition is a hallmark. The delineation of the GSS gave an important lead to the first demonstration by Prusiner of a pathogenic mutation in a PRNP gene.

The evaluation of amyloid deposition in these transmissible diseases (subsequently shown by others to be comprised of the PrP or prion protein) led in 1978 to the beginnings of a project to study the nature of the amyloid deposits in Alzheimer's disease. The amyloid was first purified from the neuritic plaques in 1979, but it was not until a collaboration was formed in 1984 with Konrad Beyreuther (then at the Institute of Genetics, Cologne, and now at the University of Heidelberg) that the N-terminal sequence of the Alzheimer plaque amyloid was obtained. The collaboration has continued to the present, resulting in the following achievements:

1978/85: Purified, sequenced and defined the aggregational properties of the amyloid Aβ in the plaques of Alzheimer's disease.

1985/86: Obtained the first evidence of oxidative stress in the Alzheimer's disease brain. This is now recognized as the effect of Aβ toxicity in the brain.

1987: Cloned (with B Müller-Hill) the amyloid precursor protein (APP) and demonstrated that the plaque amyloid is maximally 42 or 43 residues in length; chromosome 21 localised for gene for APP, and demonstrated that some familial AD pedigrees are not linked to this locus (with C van Broeckhoven).

1988/89: Demonstrated the transmembrane orientation of APP; the APP promoter cloned. Demonstrated secretion of APP from cells through a mechanism of C-terminal truncation; pathologic evolution of the plaque from diffuse deposits to dense cores; demonstrated APP over-expression in Down's syndrome and an assay for the C-terminus of APP in serum.

1990/91: Showed axonal transport of APP (with D Price); presence of APP in platelets as an important method for studying *in vivo* metabolism in humans; regulation of APP expression and splicing during differentiation. The significance of differential aggregational properties of the amyloid and solubility profiles of peptides differing by two residues at their C-termini (40 vs 42 amino acids).

1992/94: Discovered interactions of metals with amyloid aggregation (with A Bush); assay of APP in plasma in Alzheimer's disease; role of APP in neurite outgrowth; extracellular matrix binding properties of APP; purified APP from human brain; demonstrated a new alternately spliced isoform of APP in neurons. Identified a novel zinc binding site in the APP ectodomain; interactions between zinc, copper and heparin binding sites in APP and their roles in APP function; surface expression of APP on activated platelets; secretion of APP from cultured neurons; interaction of zinc in the process of amyloid aggregation (with A Bush and R Tanzi); alternate splicing of the APP-related protein (APLP-2).

1995: Purified of proteoglycans that bind APP; demonstrated intracellular Aβ production and the effect of the metalloprotease inhibitor, phosphoramidon; amyloidgenic processing of APP in platelets; extracellular matrix affects processing of APP; a cathepsin D - like enzyme involved in C-terminal processing of APP.

1996/99: Discovered major effect of dietary zinc on APP processing in rats; surface expression of APP on neurons *in vitro*; novel interactions between copper (II) and APP; effect of splicing of the juxta-membranous domains on trafficking of APP; collagen binding site on APP; methods for assaying APP and Aβ in human plasma; large scale expression and purification of APP in the yeast *Picia pastoris;* down regulation of APP causes loss of neuronal adhesion; identification of the heparin sulfated proteoglycan, glypican, as a major binding partner of APP; the axonal sorting signal of APP is localised to the Aβ domain of APP - the first identification of any axonal sorting signal; a novel metalloprotease in the Golgi membranes of brain which generates amyloidogenic fragments; distinct pathways for intracellular and secreted forms of Aβ amyloid in cultured neurons; a transgenic mouse model of Alzheimer's disease with high levels of brain Aβ expression.

These studies on Alzheimer's disease are now focused on identifying the pathways through which environmental and genetic factors can operate to cause this disease. In collaboration with the pharmaceutical industry, a multidisciplinary approach is now directed at identifying lead compounds which can inhibit the production or aggregation of amyloid in the AD brain: a new class of protease inhibitors has been identified which has very promising activity *in vitro* and will shortly be evaluated in whole-animal trials. Commercial development of compounds which are directed at the toxicity of the βA4 amyloid has commenced.

TEACHING AND ADMINISTRATIVE SERVICE:

1981-1988 Within the Department of Pathology, University of Western Australia. Supervision of postgraduate students for Honours Degree, Masters Degree, and Doctor of Philosophy.

Lectures, seminars and tutorials to medical students as part of the Pathology undergraduate curriculum and to science students in the Departments of Microbiology and Pathology.

Participant in Neuroscience undergraduate teaching program at the University of Western Australia.

1989 - present Co-ordination of Pathology teaching to Medical, Dental, Optometry, Physiotherapy and Science students at the University of Melbourne, undergraduate and postgraduate. Implementation of the New Curriculum for Medical Students.

1996 - Coordinator, Master of Medicine in Clinical Neuroscience, the University of Melbourne.

1998 - PhD Committee of the Academic Board, The University of Melbourne

Patents:

1. Title APP promoter
   Assignee: The University of Heidelberg
   Inventors: Salbaum, Beyreuther, Masters
   Status: US patent: 08/483, 488; 08/589, 316
   Canadian patent: 609, 716
   Filed: 1988

2. Title A method of assaying and treating Alzheimer's Disease.
   Assignee: The University of Melbourne (ref: -)
   Inventors: Bush, Beyreuther, Masters
   Status: International patent, PCT/AU92/00610;
   Australian patent, 29263/92;
   Canadian Patent, 2123211;
   European patent, 92923431.8;
   Japanese patent, 508824/93;
   US patent pending, 08/757,537.

3. Title APP Transgenic Animal Model
   Assignee: The University of Melbourne, SmithKline Beecham
   Inventors: Lichtenthaler, Beyreuther, Masters
   Status: UK Patent 96155351.5, 9618804.0
   Filed: 8/5/97

4. Title APP platelet aggregation inhibitors
   Assignee: The University of Melbourne
   Inventors: Henry, Cappai, Beyreuther, Masters
   Status: UK patent 9715266.4, 9715269.8
   Filed: 18/7/97

Colin L. Masters, MD

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1970

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1975

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1976

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1987

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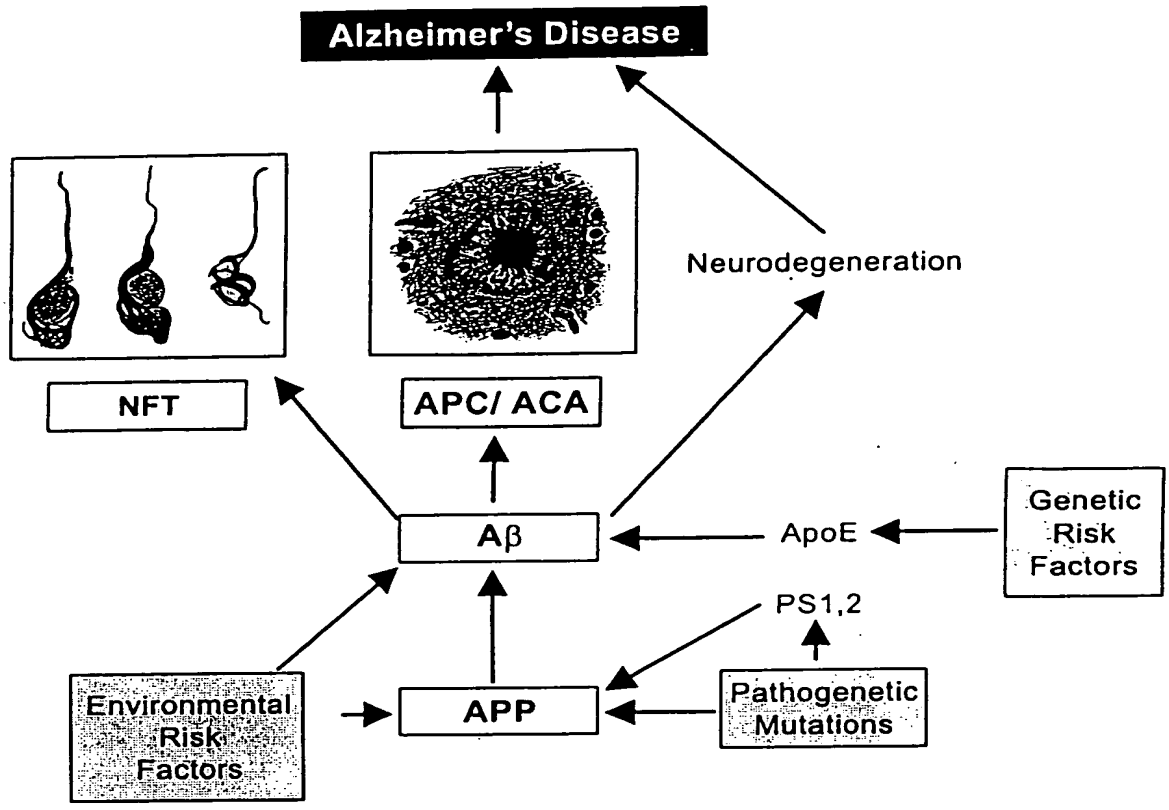
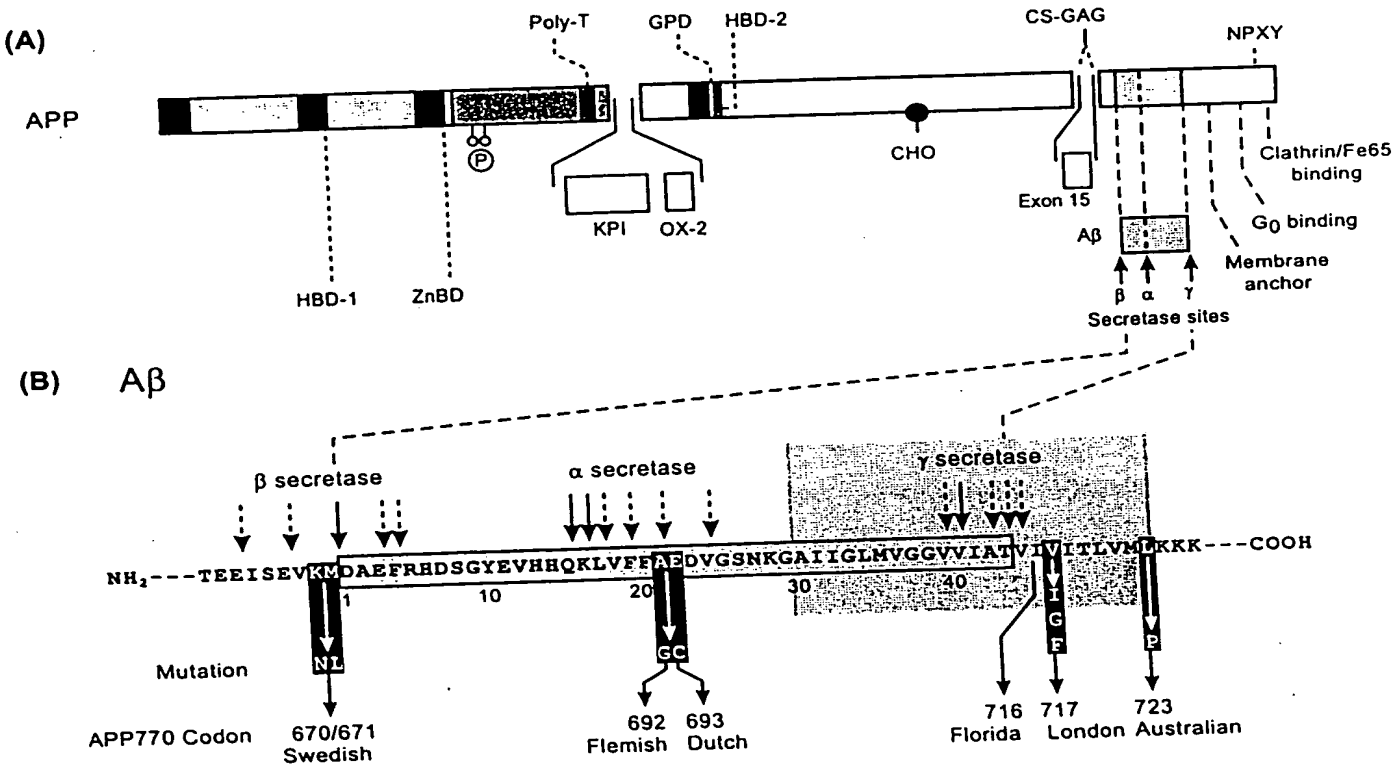
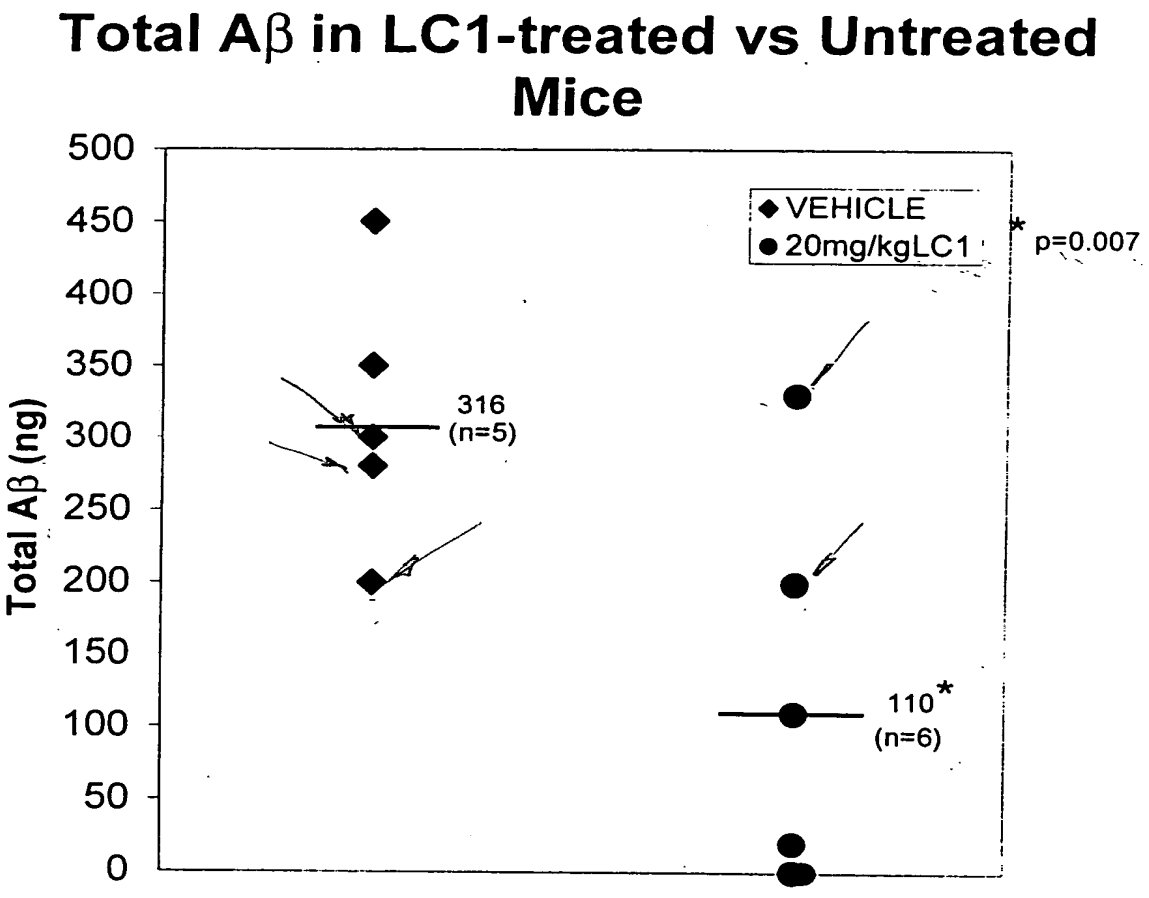
The Amyloidocentric Pathways in Alzheimer's Disease



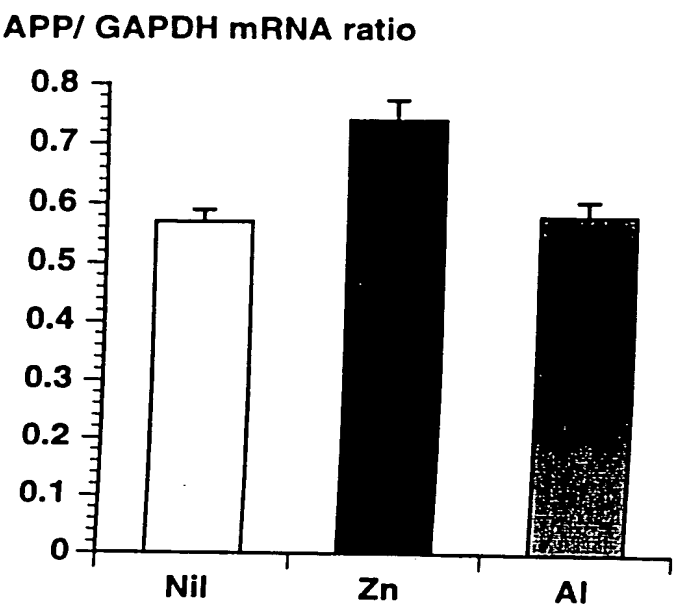

(A)
APP
Poly-T
GPD
HBD-2
CS-GAG
NPXY
CHO
Exon 15
Clathrin/Fe65 binding
KPI
OX-2
Aβ
G0 binding
Membrane anchor
HBD-1
ZnBD
β α γ
Secretase sites
(B) Aβ
β secretase
α secretase
γ secretase
NH2---TEEISEVKMDAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIATVIVITLVMLKKK---COOH
1
10
20
30
40
Mutation
APP770 Codon
670/671 Swedish
692 Flemish
693 Dutch
716 Florida
717 London
723 Australian


Total Aβ in LC1-treated vs Untreated Mice
Total Aβ (ng)
500
450
400
350
300
250
200
150
100
50
0
VEHICLE
20mg/kgLC1
*
p=0.007
316
(n=5)
110*
(n=6)

Effects of zinc supplementation upon rat brain APP mRNA. APP mRNA is expressed as a ratio to GAPDH (from the same sample), for the combined control groups (n=24), animals given zinc (n=12) and animals given aluminium (n=12). Error bars refer to the standard error of the mean. (* $p<0.01$, NS = $p>0.01$, Mann-Whitney U test. Kruskal Wallis ANOVA $p<0.05$). **Results are characteristic of n=4 experiments.**

METHODS Male Sprague-Dawley rats between 6 to 12 months of age, were isolated and given either double-distilled water (ddH_20) or ddH_20 with added heavy metals (average of three zinc experiments; 0.245 ± 0.0.007 g/L Zn as $ZnCl_2$ [measured by colorimetric assay. Randox. Antram, UK]; and 0.369 ± 0.005 g/l Al as $Al_2(SO_4)_3$. [measured by atomic absorption mass spectroscopy]). The animals were allowed free access to normal feed and ddH_20 (as above); their water consumption and body weight measured at the end of the study. During the study all the animals appeared well, and there were no differences in animal weights at the completion of the study. In animals given aluminum supplementation there was a slight significant reduction ($p<0.05$, Mann-Whitney U test) in water intake (0.44 ± 0.08 ml/g body weight) compared to the aluminum controls (0.58 ± 0.08 ml/g body weight). Each metal-treatment group was compared to a separate control group (of equal number) and processed together.
After seven days the animals were sacrificed and equal weights of rat cerebral hemispheres were homogenised in ice-cold homogenisation buffer

Total RNA was extracted by the RNAzol method (Biotec Laboratories, Houston, Texas), and 20ug/lane of total RNA was prepared by precipitation, and then resuspended in DEPC treated water and formamide sample buffer. Heat denatured samples were electrophoresed on a 1.2% agarose gel with 6.7% formaldehyde and northern blotted; RNA fixed to the membrane by heating in an 80 °C oven for 30 minutes. A portion of APP_{770} sequence (nucleotides 1795 to 2853) corresponding to a segment of DNA encoding most of the βA4 domain, continuing past its stop codon, was excised with Eco RI; purified from an agarose gel, radiolabelled by random priming, and then purified from unincorporated nucleotides. The membrane was prehybridised in hybridisation buffer at 65°C for at least two hours, and then hybridised in buffer containing at least 10^6 cpm/ml of heat denatured labelled probe, for 16 hrs at 65°C. The filters were washed in 0.2 × SSC / 0.25% sarcosine (2 × 30 minutes at 65°C) and exposed to a FUJI Bas 1000 phosphoimager screen for 4-24 hours. Screens were read using a FUJI Bas phosphoimager and signals quantified using the Bas 1000 software.


APP/ GAPDH mRNA ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Nil
Zn
Al

(99.17) In Press J Biol Chem

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Aqueous dissolution of Alzheimer's disease Aß amyloid deposits by biometal depletion

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Running Title: Alzheimer amyloid is dissolved by chelators

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Abbreviations

BC	Bathocuproine disulfonic acid
EGTA	Ethylene glycol –bis(ß-aminoethyl ether)-N,N,N',N' - tetra acetic acid
TPEN	N,N,N',N'–tetrakis(2-pyridyl-methyl) ethylene diamine
EDTA	Ethylene (dinitrilo) tetra -acetic acid
SDS	Sodium dodecyl sulfate
BCA	Bicinchoninic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline

SUMMARY

Zn(II) and Cu(II) precipitate Aß *in vitro* into insoluble aggregates that are dissolved by metal chelators. We now report evidence that these biometals also mediate the deposition of Aß amyloid in Alzheimer's disease, since the solubilization of Aß from post mortem brain tissue was significantly increased by the presence of chelators- EGTA, TPEN and bathocuproine. Efficient extraction of Aß also required Mg(II) and Ca(II). The chelators were more effective in extracting Aß from Alzheimer's disease brain tissue than age-matched controls, suggesting that metal ions differentiate the chemical architecture of amyloid in Alzheimer's disease. Agents that specifically chelate Cu and Zn ions, but preserve Mg(II) and Ca(II), may be of therapeutic value in Alzheimer's disease.

INTRODUCTION

Aß is the main component of the amyloid deposits that characterise the neuropathologic lesions of Alzheimer's disease (AD). The mechanism leading to the precipitation of this normally soluble protein is unknown, but is related to the pathogenesis of the disorder since all mutations linked to familial AD alter Aß structure or metabolism (1), and the deposition of ß-amyloid in the neocortex of transgenic mice overexpressing Aß is accompanied by most of the other neuropathological features of AD (2). We have previously found that Zn(II), Cu(II) and, to a lesser extent, Fe(III), at low μM concentrations, induce the rapid aggregation of synthetic Aß (3). These transition metal ions are highly concentrated in the neocortical regions most affected in AD, and all three metal ions are both significantly elevated in the neuropil of these regions in Alzheimer's disease, and further concentrated within amyloid plaque deposits (4).

We recently reported that Zn(II)- or Cu(II)- induced Aß precipitation is reversed by treating the aggregate with metal chelators (5-6). We hypothesized that if the metal ions within brain amyloid mediated the assembly of Aß aggregates, then treating tissue with metal chelators should induce the solubilization of Aß. We tested this hypothesis by extracting Aß amyloid-bearing post mortem brain tissue in the presence and absence of various metal ion chelators and assaying the distribution of Aß within the soluble and insoluble phases.

EXPERIMENTAL PROCEDURES

Tissue selection

Post-mortem tissues, stored at -80°C, were obtained from the NH&MRC supported Brain Bank at the University of Melbourne, together with accompanying histopathological and clinical data. AD was assessed according to CERAD criteria (7). In order to examine the chemical architecture of the Aß deposition that is observed in non-AD aged brain, Aß immunohistochemistry was used to select age-matched control (AC) cases that did not reach CERAD criteria, and in which amyloid deposition, if present, was detectable only in the form of diffuse plaques, but not neuritic plaques.

Selection of chelators

No available chelator is exclusively specific for any particular metal ion, therefore we surveyed the effects of chelators that display various respective affinities for zinc and/or copper ions relative to more abundant metal ions such as calcium and magnesium. The pKa values of N,N,N^1,N^1-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN) are: Al(III)= negligible, Ca(II) = 3, Cu(II) = 20.2, Fe(III) = 14.4, Mg(II) = negligible, Zn(II) = 15.4; EGTA are: Al(III) = 13.9, Ca(II) = 10.9, Cu(II) = 17.6, Fe(III) = 11.8, Mg(II) = 5.3, Zn(II) = 12.6; bathocuproine (BC): Al(III) = negligible, Ca(II) = negligible, Cu(II) = 6.1, Cu(I) = 19.1, Fe(III) = negligible, Mg(II) = negligible, Zn(II) = 4.1 (ref 8).

Sample preparation

The cortical meninges were removed and gray matter (0.5 g) was homogenised using a DIAX 900 homogeniser (Heidolph & Co, Kelheim, Germany) for 3 x 30s periods at full speed, with a 30s rest between strokes, in 3 ml of ice-cold phosphate-buffered saline ("PBS"), pH 7.4, containing a mixture of protease inhibitors (BioRad, Hercules, CA), with the exception of EDTA, or in the presence of either various chelators or metal ions prepared in PBS. To obtain the PBS-extractable fraction, the homogenate was centrifuged at 100,000 x g for 30 min, the supernatant removed and divided into 1 ml

aliquots. Protein within a 1ml supernatant sample was precipitated using 1:5 ice-cold 10% trichloracetic acid (TCA), and pelleted by centrifugation at 10,000 x g for 20 mins. The pellet was prepared for PAGE by boiling for 10 min in Tris-tricine SDS-sample buffer containing 8% SDS, 10% mercaptoethanol and 8M urea. Total Aß in the cortical samples was obtained by homogenizing in 1 ml PBS and boiling in sample buffer as above.

PAGE and Western blotting

Tris-tricine PAGE was performed by loading samples onto 10 well, 10-20% gradient gels (Novex, San Diego, CA), followed by transfer onto 0.2 mm nitrocellulose membrane (BioRad, Hercules, CA). The Aß was detected using monoclonal antibodies WO2 (which detects Aß40 and Aß42 at an epitope between 5-8), G210 (which is specific for Aß species that terminate at carboxyl residue 40) or G211 (which is specific for Aß species that terminate at carboxyl residue 42) (9), in conjunction with HRP-conjugated rabbit anti mouse IgG (Dako, Denmark) and visualised using chemiluminescence (ECL, Amersham Life Science, UK). Each gel included two or more lanes containing known quantities of synthetic Aß (Keck Laboratory, Yale University New Haven, CT) as reference standards.

Blot scanning and transmission densitometry assay for Aß.

Blot films were scanned using a Relisys scanner with transparency adapter (Teco Information Systems, Taiwan) and densitometry performed using Image 1.6 software (NIH, Bethesda, MD). The dynamic range of the film/scanner was determined using a step tablet (No. 911ST600 , Kodak, Rochester NY), a calibrated film exposed by the manufacturer to provide steps of known increasing intensity. The quantifiable range of signal intensity for densitometric analysis of our Aβ bands was based on the comparison with a curve obtained by scanning and densitometry of the step tablet. The dynamic range of the scanner was increased by using a transparency adapter rather than reflection.

For the survey comparing levels of Aß in post-mortem brain samples from AD cases and controls

(Fig. 3), the combined signals generated from 4.3 kDa immunoreactive Aß (apparent monomer) and 8.6 kDa immunoreactive Aß (apparent dimer) were quantified. Successive ECL exposure times of 2 min, 5 min, 10 min, 15 min and 30 min were routinely performed to establish the optimal exposure for each individual blot, so that the relative amounts of Aß measured by transmission densitometry remained in the linear response range of the assay, while determining at what point the signal from the Aß standards had reached saturating intensity. Preliminary blots were routinely performed to determine how the samples were to be subsequently diluted so as to try to ensure that the Aß signals fell within the quantifiable portion of the Aß standard curve. All the experimental samples extracted from the same brain specimen were initially diluted to the same degree and included on the same blot for analysis (as in Fig. 3A). However, it was usually not possible to determine all the Aß readings from one blot at one dilution. The Aß content varied broadly between the extracted samples (note the range in Aß intensity between the various extracts of the same brain specimens illustrated in the blots in Fig. 3A), and therefore it was usually necessary to perform subsequent individual blots on specific samples that had been further diluted so as to generate Aß signals that fell within the linear range of the standard curve.

This technique was chosen for Aß assay in preference to ELISA since it has the advantage of discriminating the M_r of the Aß immunoreactivity and therefore is less likely to inappropriately detect non-Aß species such as APP fragments, like those that have recently been found to have been inadvertently cross-reacting with Aß in an assay that previously had been considered to be well-characterized (10)

The efficiency of the TCA precipitation procedure was validated by testing samples of whole human serum diluted 1:10 to which had been added 2mg of synthetic Aß 1-40 or Aß 1-42. Aß recovery was assessed by extracting the precipitate into SDS sample buffer and performing Western blot analysis against synthetic Aß standards as above. Protein in the TCA pellet was estimated by resuspending the pellet in water and assaying the protein recovery using a BCA assay (Pierce, Rockford, IL). This indicated that the efficiency of protein and Aß precipitation was approximately 90%. The efficiency of the 8M urea solubilization was found to be more efficient and less variable that of formic acid in a

parallel, blinded assay conducted independently. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated.

Analysis of metals

The post-centrifugation pellets were dissolved in 4 ml x 3N HNO3+1N HCl for 24 hours and then assayed by inductively-coupled plasma atomic emission spectroscopy (ICP-AES).

RESULTS

AD frontal cortex was compared to tissue from the same region of age-matched controls (AC). A survey of the effects of the chelators at a range of concentrations (0-5 mM) on six AD cases confirmed that the solubilization of Aß was specifically enhanced by the presence of chelator (Figure 1), although total TCA-precipitable protein was not affected by any of the chelators at the concentrations tested (data not shown).

Extraction of AD brain into PBS alone liberated a small amount of Aß into the soluble phase in every case, confirming previous reports (11-14). In contrast, homogenization in the presence of either EGTA and TPEN at concentrations between 0.004 mM and 0.1 mM, significantly increased soluble Aß extraction. The optimum concentrations of EGTA or TPEN for the resolubilization of Aß varied considerably from case to case, and did not show linear concentration dependency. Typically, as illustrated in Figure 1A, there was a biphasic response in Aß extraction as concentrations of EGTA or TPEN were increased. One peak typically occurred when homogenization was performed in the presence of 0.004 mM of either chelator. A second peak in Aß soluble extraction occurred at about 0.1 mM (for EGTA) and 2 mM for TPEN (although there was considerable case-to-case variation and the case illustrated in Fig. 1A had an extraction peak in response to 0.1 mM TPEN). Both TPEN and EGTA were less effective at extracting Aß when present at concentrations in the millimolar range, and EGTA at ≥2mM abolished the signal for Aß (Fig 1B). In contrast, BC elicited a concentration-dependent increase in Aß extracted from AD tissue (Fig. 1C) plateauing at 10mM. This finding is of interest because BC is highly selective for Cu(I), and the result is compatible with our recent finding that Aß rapidly binds and reduces Cu(II) to Cu(I) (6), suggesting that a proportion of Aß assembly is mediated by Cu(I).

Insulin degrading enzyme (IDE), a zinc-metalloproteinase, has been reported to cleave Aß in the brain and in biological fluids (15). To determine whether chelator-mediated augmentation of Aß solubilization was due to inhibition of this enzyme, we also performed homogenizations in the presence of 1mM N-ethyl maleimide, a potent inhibitor of IDE. Enhancement of Aß signal was not observed

above that of PBS alone (data not shown). To determine whether other enzymatic activities may be artefactually modifying the data, we compared extraction of the brain Aß at 4 °C to extraction at 37 °C. There was no decrease in Aß signal to suggest enhanced degradation at the higher temperature. These controls suggest that inhibition of Aß-cleaving enzymatic activities by chelators does not contribute to the generation of soluble Aß under these conditions.

To characterize the metal ions participating in the precipitation of brain-derived Aß, and to investigate the non-linear response of Aß extraction in the presence of EGTA or TPEN, we added additional metal ions to the extraction system. The presence of Cu(II) or Zn(II) in the PBS homogenization buffer abolished the increased extraction of Aß caused by chelator treatments (data not shown). Also, the presence of additional Zn(II) (≥5 μM) or Cu(II) (≥50 μM) in the homogenization buffer without chelator, abolished extraction of Aß due to treatment with PBS alone (Figure 2A). Therefore, these metal ions can modulate the solubility of Aß in this system.

The presence of Cu(II) at 5 μM in the PBS homogenization buffer without chelator increased the extraction of Aß by PBS (Fig. 2A). At pH 7.4, Zn(II) induces far more Aß aggregation than Cu(II), hence this result may be due to Cu(II) displacing Zn(II) from Aß. At 20 μM, Cu(II) induces the appearance of an apparent SDS-resistant Aß dimer, which may be due to an oxidative modification of the peptide or may represent an intermediate produced during the process of Aß aggregation.

Because millimolar concentrations of TPEN or EGTA unexpectedly suppressed Aß resolubilization, we suspected that Mg(II) or Ca(II) may participate in the resolubilization of Aß. Mg(II) and Ca(II) are more abundant than Cu(II) and Zn(II) in brain samples. Therefore, given the relative affinities of the chelators used, sequestration of Mg(II) and Ca(II) would require higher chelator concentrations than those necessary to complex Zn(II) and Cu(II). Samples of frontal cortex (0.5g) from AD were homogenised in 2 mM EGTA, a condition which consistently abolishes the solubilization of Aß (see Figure 3) while removing Zn(II), Cu(II) and other metal ions from the solid phase of the homogenate. The homogenates were centrifuged at 100,000 x g for 30 min and the supernatants discarded. The remaining (metal depleted) pellets were rehomogenised in a further 2 ml of

either PBS, pH 7.4 alone, 2 mM $MgCl_2$ in PBS, or 2 mM $CaCl_2$ in PBS and the homogenates subjected to centrifugation again at 100,000 x g. Aß in the soluble fraction was visualised by Western blot with W02 as described. When Mg(II) (2 mM) or Ca(II) (2 mM) were added to the homogenisation buffer there was no appreciable alteration in the extraction of soluble Aß (data not shown). However, when supplemented to the pellet fraction of a brain homogenate previously depleted of metals by treatment with 2 mM EGTA during homogenization, Mg(II), and to a lesser extent Ca(II), both resolubilized the sedimentable Aß (Fig. 2B). Taken together, these data indicate that although removal of metal ions like Zn(II) and Cu(II) may be necessary for the resolubilization of Aß deposits, the presence of Mg(II) and Ca(II) are required for the sedimentable Aß to resolubilize. Therefore, the optimal chelator concentration for the resolubilization of Aß deposits depends upon an interplay of antagonistic factors, which may explain the non-linear response of Aß extraction to increasing chelator concentrations (Fig. 1A, B), and the case-to-case variability of the chelator concentrations required to achieve maximal extraction of Aß.

In order to investigate which metal ions are removed by chelator treatments, we measured the amounts of various metals (Al, Fe, Mg, Ca, Cu, Zn) remaining in the brain pellet after treatment with PBS ± chelator. Analysis of the effects of 0.1 mM TPEN was performed first since this treatment induced an increase in soluble Aß in the first six AD samples analysed, and because complete complexation of Mg(II) and Ca(II) was unlikely at that concentration of chelator.

The observed increase in extractable Aß correlated with significant depletion (30%) in zinc and, to a lesser extent, copper, in each of ten AD cases examined, when compared with PBS-treated tissue. No other metal measured was significantly influenced by treatment at this concentration (Table 1). A survey of the metal content of pellets taken from AD brain homogenates (n=2) treated with the complete range of chelator concentrations described in Fig. 1, confirmed that EGTA treatment at ≥2 mM depleted (>30%) the sample of both Zn, Ca and Mg, whereas treatment with TPEN at similar concentrations depleted Zn, Cu, Ca and Fe. Measurement of metals remaining in the pellet following treatment of these samples over the range of BC concentrations studied indicated that none of the metals was depleted (data not shown). Since BC has an affinity for Cu(I) that is 13 orders of magnitude greater than for

Cu(II), the lack of detectable total Cu depletion caused by treatment with BC is not unexpected since Cu levels were relatively low in these preparations, and the proportion of Cu that exists as Cu(I) is likely to be small.

To determine the consistency of chelator effects upon Aß extraction from brain, we surveyed a larger sample of specimens using two chelator concentrations (0.1 and 2 mM), and also measured the total amount of Aß in the samples by 8M urea solubilization. After measuring the effects of treatment with the three chelators upon AD (n=9) and AC (n=8) brain samples, a significant pattern emerged (Fig 3). For AD cases, significant increases of solubilized Aß, compared to the baseline amount liberated by PBS treatment, were induced by TPEN at 2 mM (2.7-fold, $p<0.001$) and BC at 0.1 mM (2.8-fold, $p<0.005$) and at 2 mM (4.1-fold, $p<0.001$). The effects of chelators upon the release of Aß from the AC group were markedly attenuated, and therefore did not reach significance with the exception of the effect of 0.1 mM EGTA, which induced a significant increase (2-fold, $p<0.01$). These data support the possibility that Zn(II) and Cu(I) maintain the aggregated state of Aß in AD brain, but are less important in the architecture of Aß aggregates in AC. EGTA (2 mM) inhibited the extraction of Aß in both AD (decreased 80%, $p<0.001$) and AC (decreased 50%, NS) groups. This result is compatible with the extraction of Ca(II) and Mg(II) from the tissue homogenates, since these are metal ions are required for the release of Aß from deposits that have been depleted of Zn and Cu (Fig. 2). The cases analysed in Figure 3 were also assayed with reference to the total amount of Aß extracted from the individual brain specimens (Table 2). The concentration of total Aß in the AD specimens was much greater (31 µg/g) than the total amount in the AC samples (2.1 µg/g). The concentration of Aß in AD brains that was extracted by PBS alone was 0.7 ± µg/g, representing 3.1% of total Aß. The amount of Aß in AD brain extracted by a single treatment with 2 mM BC increased significantly to 1.9 ± µg/g, representing 9.6% (range 2.0 - 28.8%) of total Aß. This proportion is likely to be an underestimate of the amount of Aß that is assembled by biometals, since the result was achieved by exposing the individual brain specimens to only one brief chelator treatment. Repeated extraction cycles resulted in further Aß release, up to 50% of the starting values. We limited the highest concentration of BC to 2mM for comparison with other chelators at equimolar concentrations because our initial data (Fig. 1) indicated

that millimolar concentrations of TPEN and EGTA suppressed Aß solubilization.

Treatment of AD specimens with chelators frequently generated an apparent SDS-resistant Aß dimer (immunoreactivity migrating at approximately 8.6 kDa) that was not evident when the specimen was treated with PBS alone (Fig. 4 A). Frequently, the appearance of is 8.6 kDa Aß species was not accompanied by a proportional increase in the amount of apparent Aß monomer (Fig. 4 A). These findings are relevant because SDS-resistant dimeric forms of Aß purified from AD brain has been reported to possess increased neurotoxic properties (16). The possibility that there is a specific metal ion mediated abnormality of neurotoxic Aß dimer assembly warrants further investigation.

We analysed Western blots of brain extracts with antibodies that are specific for AßX-40 (G210) and AßX-42 (G211) (Fig. 4B), since the latter Aß sub-species is enriched in AD amyloid plaques (17). We found that treatment with BC significantly increased the solubilization of both Aß sub-species in AD samples, indicating that AßX-42, while less soluble than the more abundant AßX-40 (18) is nonetheless released by chelation of Cu(I).

DISCUSSION

These data indicate that there is a pool of Aß within the affected neocortex in AD which is held in sedimentable aggregates by metal ions, likely to be Cu(I) and Zn(II), and that these aggregates are solubilized by treatment with chelators. Mg(II) and Ca(II) were found to be essential for the release of Aß. The microanatomical site of these collections cannot be determined by our methods, but is likely to be extracellular since this is where Aß deposition in AD is readily demonstrable by morphological techniques, and because chelator treatment of AC tissue (possessing much less extracellular plaque deposit) did not release as much Aß. The possibility of the artefactual combination of cellular metal ions with soluble Aß leading to Aß precipitation as a consequence of the tissue homogenization must also be considered. However, since the precipitated fraction of Aß in AD neocortex is much greater than the soluble cellular pool, this possibility is unlikely to contribute substantially to the phenomenon that we have observed. Other recent observations detecting enrichment of zinc, copper and iron in amyloid deposits by histological means (4) support the likelihood that our observations reflect the chemical structure of Aß assembly in amyloid deposits. Aß-associated, Zn/Cu- metalloproteins apolipoprotein E (19) and alpha-2-macroglobulin (20-22), may also participate in the reactions we have described.

Our data support the development of chelator compounds as chemotherapeutic agents for AD. One previous clinical trial of a chelator compound, desferrioxamine (DFO), was reported to significantly arrest the progression of the disease (23), but no further attempts to reproduce this finding have been reported. DFO, like all chelators, is not perfectly specific for a particular metal ion, and although the DFO trial was thought to target Al(III), it is possible that the beneficial effect of the treatment was due to chelation of Fe(III), Cu(II) and Zn(II). Our current findings indicate that an ideal therapeutic to dissolve Aß amyloid would involve a compound that is relatively selective for Cu(I), Zn(II) and possibly Fe(III), does not sequester Mg(II) or Ca(II), and that coordinates metal ions in the cerebral amyloid mass but not systemically.

We have recently concluded a larger study comparing soluble and insoluble Aß in AD and AC brains, and have found a significant correlation between the PBS-extractable Aß component and disease

severity (McLean, C., Cherny, R., Bush, A.I., Masters, C.L., submitted). Although representing only a small portion of the total Aß load, an approximate three-fold difference in the levels of the most readily mobilized Aß fraction distinguished AD from non-AD in an age-matched population. The present study suggests that four- to seven-fold increases in PBS-extractable Aß can be achieved by direction chelation. At the concentrations used, this effect is observed without apparent impact upon the solubility of other proteins. We have observed that chelator concentrations as low as 4 μM were effective at resolubilizing Aß deposits from AD brain samples, which indicates that delivering an effective biometal-depleting compound to the amyloid load *in vivo* may not necessitate biologically incompatible doses. Clearly, compounds targeted to the dissolution of aggregated amyloid only have promise as therapeutic agents if the resolubilized and potentially toxic Aß can be effectively cleared from the AD brain.

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FIGURE LEGENDS

Figure 1. Release of Aß from sedimentable deposits by chelators.

Frontal cortex from an AD brain was homogenized in PBS, pH 7.4 ± increasing concentrations of (A) TPEN (upper panel), (B) EGTA (middle panel) or (C) BC (lower panel). Following centrifugation, Aß in the supernatants was visualized by Western blot using anti-Aß monoclonal antibody WO2 (lower panels), and quantified by densitometry (graphs above corresponding blots). Although there is considerable variation in the optimum chelator concentration for the maximal recovery of Aß from case to case, these data are representative of 17 AD cases.

Figure 2. The effect of metals upon the solubility of brain-derived Aß.

(A) Zn(II) and Cu(II) inhibit the solubilization of Aß by PBS extraction. Specimens of AD frontal cortex were homogenized in the presence of PBS or varying concentrations of Cu(II) (as sulfate) or Zn(II) (as sulfate). After centrifugation, Aß in the supernatants was visualized as described in Fig 1.

(B) Aß in metal-depleted deposits is liberated into the soluble phase by Mg(II) and Ca(II). Samples of AD frontal cortex were homogenized in 2mM EGTA, a condition which consistently abolishes the release of Aß (see Figs 1 and 3), and removes Zn(II), Cu(II) and other metal ions from the solid phase of the homogenate. After centrifugation, the remaining (metal depleted) pellets were treated with either PBS, pH 7.4 alone, 2mM Mg(II) in PBS or 2mM Ca(II) in PBS, and the centrifuged again. Data shown are representative of Aß in the soluble fraction of the three treated samples, visualized as in Fig 1.

Figure 3. Patterns of chelator-mediated release of brain Aß in AD and age-matched, non-AD tissue.

Post mortem samples of AD frontal cortex (n=9) and age-matched controls (n=8) were treated with

PBS, TPEN, EGTA or BC (chelators at 0.1mM and 2mM) and soluble Aß assayed by Western blot. (A) The soluble material from the seven treatment conditions of each individual case were initially compared on the same blot. An iterative process was used to arrive at the final Aß concentration for each sample (per g wet weight) that involved multiple blots quantified by densitometry with reference to two standards of synthetic Aß1-40 (1 ng and 5 ng), as well as an 8M urea extract of the starting tissue. The upper panel shows a representative blot of soluble Aß extracted from an AD case. The center panel shows a representative blot of soluble Aß extracted from a control (AC). For the purposes of the illustration, similar densities of Aß signal in both the AC and the AD cases shown were achieved by loading more sample onto the AC blot and by slightly prolonging its exposure. However, when normalized against synthetic peptide standards, the amount of Aß per g of brain sample in typical AC specimens was less than AD (see Table 2). (B) A graphical representation of the effects of chelator-mediated release of brain Aß derived from data in Table 2, which summarizes averaged (± SEM) data from the AD and AC groups, where the amount of soluble Aß extracted by the six chelator treatments is expressed as a proportion of the amount of Aß solubilized by treatment with PBS alone (normalized to 100%) for each individual case.

Figure 4. Dissection of some components of metal ion-assembled brain Aß deposits.

(A) Extraction of soluble, SDS-resistant Aß dimers by chelator treatment. Representative Western blot of the AD samples that exhibit the release of a soluble Aß dimer when treated, as in Fig 3, with 0.1 mM and 2 mM TPEN, EGTA or BC.

(B) Treatment with chelators promotes the solubilization of Aß40 and Aß42 from AD brain tissue. A representative AD specimen was divided and treated with PBS ± 5mM BC, or 8M urea to estimate total Aß content (T). Western blots of the extracts were probed with monoclonal antibodies WO2 (raised against residues 5-16, recognising many Aß sub-species including Aß40 and Aß42), G210 (raised against residues 35-40, recognising Aß40), or G211 (raised against residues 35-42, recognising Aß42).

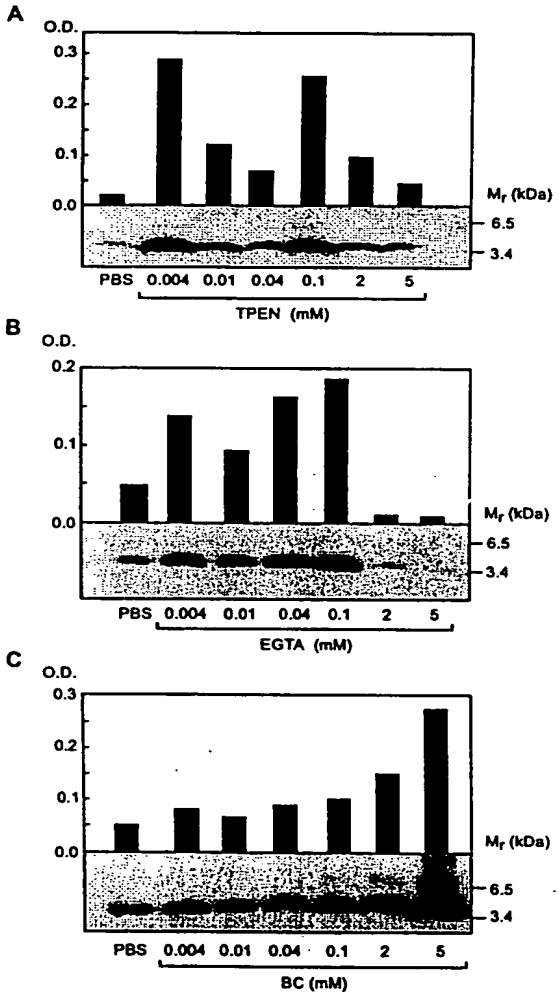

Figure 1

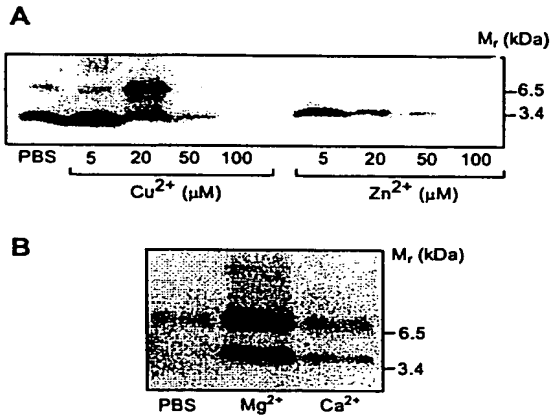

Figure 2

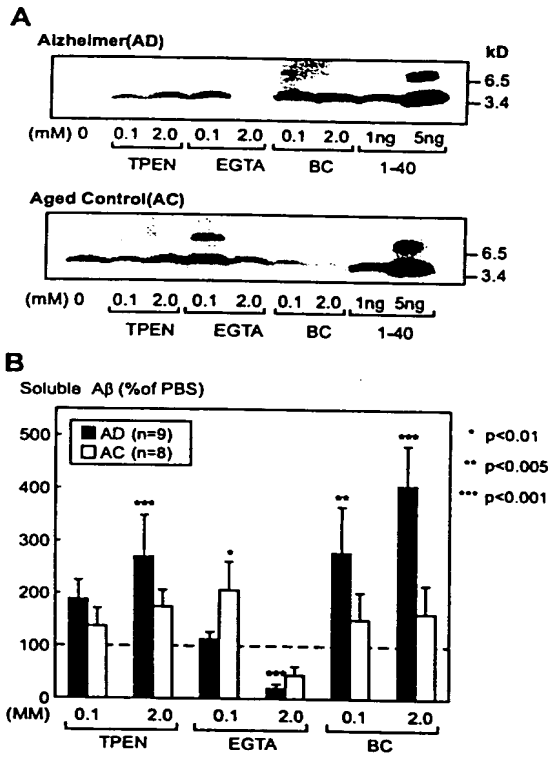

Figure 3

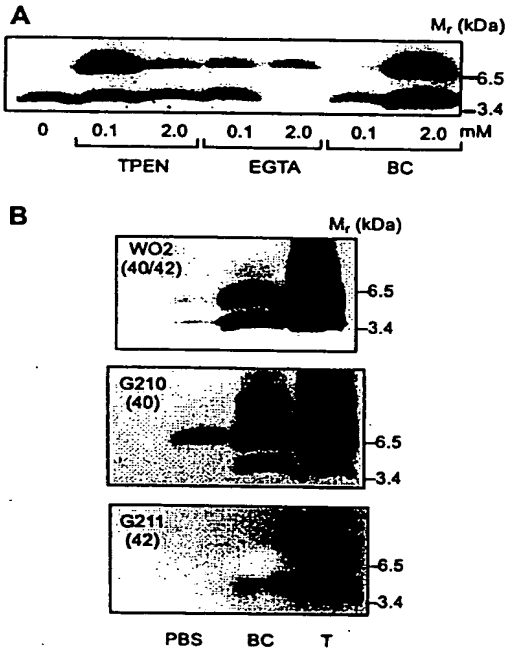

Figure 4

Table 1. Residual metal levels following treatment of brain homogenates with TPEN.

Frontal cortex from AD (n=10) was homogenised in the presence or absence of 0.1 mM TPEN and metal levels in post-centrifugation pellets were determined using ICP-AES, normalized for the starting wet weight of the tissue sample. Asterisk indicates significant difference from treatment with PBS alone on t-test ($p<0.01$).

	Zn	Cu	Fe	Ca	Mg	Al
PBS±SE	50.7±4.9	11.9±1.5	227±28.8	202±28.3	197±39.1	44.0±46.2
TPEN±SE	33.2±4.1*	9.8±1.7	239±31.7	210±37.0	230±39.2	65.0±45.0

Table 2. Concentrations of Aß extracted by PBS, chelator and 8M urea treatment of AD and AC specimens.

See legend of figure 3. Concentrations of Aß (normalized for specimen wet weight) extracted from AD and AC specimens by 8M urea (representing estimated "total" Aß content), PBS, and two chelators (0.1 and 2.0 mM), were compared. To illustrate the variation, data from each case is shown.

AD subject	1	2	3	4	5	6	7	8	9	X ±SEM
Total Aß (µg/g)	22	77	12	80	15	24	8	33	10	31 ±9.3
PBS (µg/g)	0.2	1.3	0.1	0.3	0.9	0.5	0.4	1.6	0.6	0.7 ±0.2
(% of total)	(0.9)	(1.7)	(0.8)	(0.4)	(6.0)	(2.1)	(5.0)	(4.8)	(6.0)	(3.1 ±0.8)
TPEN 0.1mM (µg/g)	0.6	2.8	0.4	0.3	0.8	0.9	0.8	1.6	0.7	1.0 ±0.3
(% of total)	(2.7)	(1.7)	(3.4)	(0.4)	(5.3)	(3.8)	(10)	(4.8)	(7.0)	(4.3 ±0.9)
TPEN 2mM (µg/g)	0.3	1.8	0.9	0.8	2.0	1.3	1.2	2.9	0.5	1.3 ±0.3
(% of total)	(1.4)	(2.3)	(7.5)	(1.0)	(13)	(5.4)	(15)	(8.8)	(5.0)	(6.6 ±1.7)
EGTA 0.1mM (µg/g)	0.3	1.5	0.04	0.5	1.1	0.5	0.7	1.7	0.4	0.8 ±0.2
(% of total)	(1.4)	(1.9)	(0.3)	(0.6)	(7.3)	(2.1)	(8.8)	(5.2)	(4.0)	(3.5 ±1.0)
EGTA 2mM (µg/g)	0.1	0.8	0	0	0.2	0	0.1	0.5	0.1	0.2 ±0.1
(% of total)	(0.5)	(1.0)	0	0	(1.3)	0	(1.3)	(1.5)	(1.0)	(0.7 ±0.2)
BC 0.1mM (µg/g)	0.14	1.8	0.9	1.4	1.2	0.9	1.6	3.1	0.5	1.3 ±0.4
(% of total)	(0.6)	(2.3)	(7.5)	(1.8)	(8.0)	(3.8)	(20)	(9.4)	(5.0)	(6.5 ±1.9)
BC 2mM (µg/g)	0.8	3.3	0.8	1.6	1.7	1.5	2.3	3.7	1.5	1.9 ±0.3
(% of total)	(3.6)	(4.3)	(6.7)	(2.0)	(11.3)	(6.3)	(28.8)	(11.2)	(15.0)	(9.6 ±2.7)

Aged Control subject	1	2	3	4	5	6	7	8	X ±SEM
Total Aß (µg/g)	0.7	0.5	1.0	4.2	2.7	3.2	3.6	0.5	2.1 ±0.52
PBS (µg/g)	0.17	0.16	0.03	0.13	0.18	0.11	0.66	0.06	0.19 ± 0.07
(% of total)	(24)	(32)	(3.0)	(3.0)	(6.7)	(3.4)	(18.3)	(12)	(12. 8 ±3.9)
TPEN 0.1mM (µg/g)	0.12	0.17	0.10	0.29	0.10	0.10	0.60	0.08	0.19 ±0.07
(% of total)	(17)	(34)	(10)	(6.9)	(3.7)	(3.1)	(16.7)	(16)	(13.4 ±3.7)
TPEN 2mM (µg/g)	0.22	0.17	0.10	0.38	0.26	0.09	1.1	0.09	0.30 ±0.12
(% of total)	(31)	(37)	(10)	(9.0)	(9.6)	(2.8)	(30)	(18)	(18.4 ±4.5)
EGTA 0.1mM (µg/g)	0.39	0.22	0.17	0.28	0.15	0.12	1.0	0.10	0.30 ±0.1
(% of total)	(55.7)	(44)	(17)	(6.7)	(5.5)	(3.8)	(27.8)	(20)	(22.6 ±7.0)
EGTA 2mM (µg/g)	0.15	0.03	0.03	0	0	0.04	0.2	0	0.06 ±0.03
(% of total)	(21.4)	(6.0)	(3.0)	(0)	(0)	(1.25)	(5.5)	(0)	(4.6 ±2.7)
BC 0.1mM (µg/g)	0.09	0.15	0.15	0.20	0.18	0.08	0.98	0.08	0.23 ±0.11
(% of total)	(12.9)	(30)	(15)	(4.8)	(6.7)	(2.5)	(27.2)	(16)	(14.3 ±3.7)
BC 2mM (µg/g)	0.03	0.04	0.15	0.24	0.30	0.08	1.16	0.10	0.26 ±0.14
(% of total)	(4.3)	(8.0)	(15)	(5.7)	(11)	(2.5)	(32)	(20)	(12.3 ±3.5)

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